Mathematical and computational modelling of post-transcriptional gene relation by micro-RNA

Mathematical and computational modelling of post-transcriptional gene regulation by microRNAs

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Abstract

Mathematical models and computational simulations have proved valuable in many areas of cell biology, including gene regulatory networks. When properly calibrated against experimental data, kinetic models can be used to describe how the concentrations of key species evolve over time. A reliable model allows 'what if' scenarios to be investigated quantitatively *in silico*, and also provides a means to compare competing hypotheses about the underlying biological mechanisms at work. Moreover, models at different scales of resolution can be merged into a bigger picture 'systems' level description. In the case where gene regulation is post-transcriptionally affected by microRNAs, biological understanding and experimental techniques have only recently matured to the extent that we can postulate and test kinetic models. In this chapter, we summarize some recent work that takes the first steps towards realistic modelling, focusing on the contributions of the authors. Using a deterministic ordinary differential equation framework, we derive models from first principles and test them for consistency with recent experimental data, including microarray and mass spectrometry measurements. We first consider typical misexpression experiments, where the microRNA level is instantaneously boosted or depleted and thereafter remains at a fixed level. We then

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move on to a more general setting where the microRNA is simply treated as another species in the reaction network, with microRNA-mRNA binding forming the basis for the post-transcriptional repression. We include some speculative comments about the potential for kinetic modelling to add to the more widespread sequence and network based approaches in the qualitative investigation of microRNA based gene regulation. We also consider what new combinations of experimental data will be needed in order to make sense of the increased systems-level complexity introduced by microRNAs.

1 Introduction

Most computational efforts to understand post-transcriptional gene regulation by microRNAs (miRNAs) have focused on target prediction tools, as reviewed by Rajewsky (2006), and related algorithms, tools and databases are becoming readily available; see, for example, (Xiao et al. 2008). In this chapter, we deal instead with mathematical and computational modelling of gene regulation by miRNAs. We overview the current state of the art, focusing on our own contribution to the field, and speculate on the role of the quantitative modelling in understanding the mechanisms and functions of miRNAs in health and disease. The motivation behind this chapter stems from the need to develop comprehensive models of gene regulation on both transcriptional and post-transcriptional levels with the goal of further integration of such models with target prediction algorithms in the overall complex regulatory network of genes, proteins and RNAs.

The first papers on post-transcriptional gene regulation were proposed in (Levine et al. 2007a, Shimoni et al. 2007, Levine et al. 2007c), and focused on the regulation by small RNA (sRNAs). A quantitative two-class model of gene regulation in *E. coli* (Levine et al. 2007a) demonstrated that sRNAs provide a novel mode of gene regulation with a threshold-linear response, a robust noise resistance characteristic, and a built-in capability for hierarchical cross talk. It has also been shown quantitatively that regulation by sRNAs is advantageous when fast responses to external signals are needed and that regulation by sRNA may provide fine-tuning of gene expression (Shimoni et al. 2007). In addition, sRNAs have been suggested to participate in sharpening a gene expression profile that was crudely established by a morphogen (Levine et al. 2007c).

MiRNAs function very much like small interfering RNAs. However, they are distinguished by their distinct pathway for maturation and by the logic through which they regulate gene expression (Du & Zamore 2005). Coupled degradation of target mRNA and its regulator (Levine et al. 2007a) is specific to gene regulation by sRNAs. By contrast, miRNAs, which are incorporated into the RISC complex, do not degrade with their targets but return to the cytosol to begin a new round of target mRNA repression. It is plausible, however, that due to increased endonucleolytic activity, miRNA may be degraded after a few cycles of target mRNA binding (Levine et al. 2007b).

Several kinetic models of gene regulation by sRNAs, and miRNAs in particular, have recently been published. Before reviewing the kinetic models of gene regulation by miRNAs, we outline some significant experimental findings that guide the modelling process.

It is well established that miRNAs regulate gene expression post-transcriptionally, influencing stability, compartmentalization and translation of mRNAs (see Figure 1). The underlying molecular mechanisms are still debated (Filipowicz et al. 2008) but the overall effect of miRNA appears to be repressive. It has been computationally predicted (Krek et al. 2005) and demonstrated experimentally (Lim et al. 2005, Baek et al. 2008, Selbach et al. 2008) that one type of miRNA may regulate large number, sometimes in the hundreds, of different types of target mRNAs and proteins.

The regulating effect of miRNAs is typically studied by conducting miRNA mis-expression experiments and measuring gene expression with microarrays (Lim et al. 2005), protein levels by powerful mass spectromic method SILAC (Baek et al. 2008) and changes in protein synthesis by pulsed SILAC (pSILAC) (Selbach et al. 2008). There are two general types of miRNA mis-expression experiments (Baek et al. 2008, Krützfeldt et al. 2005, Selbach et al. 2008),

1. overexpression or transfection of a miRNA to a cell-line or tissue where it is initially not present, or present at small levels, and

2. knock-down of an abundant miRNA by antagomir or LNA.

Recent proteomic and microarray studies have isolated the major sequence determinant that mediates miRNA regulation of both mRNAs and protein: the 6-mer "seed" (Watson-Crick consecutive base pairing between mRNAs

and the miRNA at position 2-7 counted from its 5' end) located in the 3' untranslated regions (3'UTRs) of mRNAs (Selbach et al. 2008). The accuracy of target prediction algorithms can be improved by allowing for evolutionary conservation of the seed site (Selbach et al. 2008). However, the false-positive rate of target predictions, even with conserved seed incorporation, is still estimated at 40% (using the entire pSILAC dataset). The extent of the miRNA-mediated regulation is relatively mild and depends on the number of seeds and the distances between them. It also is affected by by many other factors, including the sequence elements around the seed, sites for RNA-binding proteins and secondary structure. This is a subject of intensive research. Availability of mRNA expression levels from miRNA mis-expressions and various tissues as well as protein levels as measured by SILAC (Baek et al. 2008) and protein production as measured by pSILAC (Selbach et al. 2008) gives an opportunity to improve existing targets prediction algorithms (Friedman et al. 2008).

MiRNAs are thought to act by binding to their target mRNAs rather than through a catalytic mechanism requiring only a transient association between the miRNA and mRNA (Doench & Sharp 2004). The bulk of miRNAs are associated with target mRNAs undergoing translation (Maroney et al. 2006). It is reasonable to assume that the miRNA:mRNA complexes are being translated, but at a slower rate than free mRNAs. Certain mRNAs accumulate in P-bodies (PB) in a microRNA-dependent manner (Bhattacharyya et al. 2006), raising the possibility that PBs might be involved in miRNA-mediated repression. However, microRNA-mediated repression is unaffected in cells devoid of microscopically visible PBs, suggesting that PB formation itself is not required for repression. This led (Leung & Sharp 2007) to conclude that PBs are a consequence, not the cause, of the microRNA-mediated silencing.

Just as there are unanswered questions relating to the sequence characteristics of each miRNA:mRNA pair, relatively little is known about how the extent of regulation depends on the expression levels the miRNA, the target of interest as well as other targets that are affected by that miRNA. This is an area where modelling and computation hold great promise for adding value to experimental data sets. MiRNA mis-expression data can be used to infer kinetic parameters for various processes involved and to elucidate the mechanisms of miRNA-mediated target regulation. Other problems that can be addressed include estimating the time-response of the system to miRNA transfection and knock-down (de-repression). As more data, including miRNA studies by proteomics, becomes available, there is further

potential to use dynamical models to add a quantitative level of understanding that can be used to test hypotheses and tackle 'what if' scenarios.

2 A simple model of miRNA-mediated gene regulation

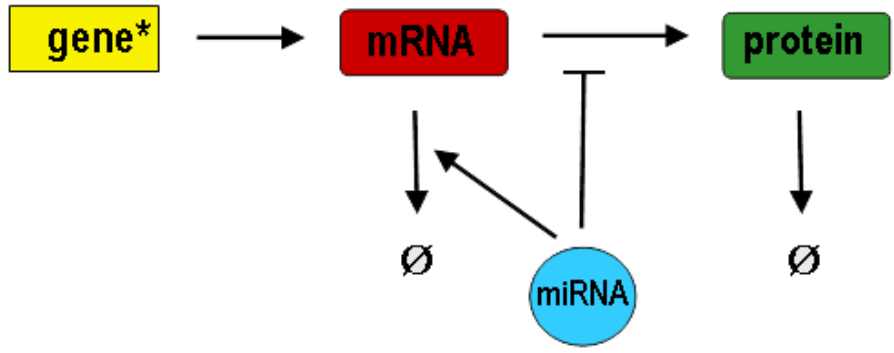


Figure 1: MicroRNA exerts its downregulating effect on mRNA or/and protein.

We now discuss a simple yet plausible model of target regulation by miRNA. The model summarizes the overall effect of miRNA on a target, without representing specific molecular mechanisms of the downregulation. This model assumes that the miRNA level remains fixed—see section 5 for a discussion of the more general case where kinetics for miRNA are introduced.

Gene expression, or mRNA levels, m, and protein levels, p, change through production and degradation (Bolouri & Davidson 2002). We may describe these rates of change with an ordinary differential equation (ODE)

$$\frac{dm(t)}{dt} = q(t) - \delta \cdot m(t), \tag{1}$$

$$\frac{dp(t)}{dt} = \lambda \cdot m(t) - \delta_P p(t). \tag{2}$$

In models of transcriptional regulation it is commonly assumed that while the production rate governing mRNAs, $q(t)$, depends on the availability of a single, or multiple, transcription factors (TFs), the mRNA degradation and protein translation are first-order processes that occur with constant rates, δ and λ, respectively. We also assume a constant protein degradation rate, δ_P.

When a transcript is a target of a specific miRNA, its degradation rate, δ, and its rate of translation, λ, both depend on the levels of this miRNA: $\delta = \delta(\texttt{miRNA})$ and $\lambda = \lambda(\texttt{miRNA})$. It is known that the presence of miRNA enhances the degradation rate (Krützfeldt et al. 2005, Lim et al. 2005) and inhibits translation (Ambros 2004, Jackson & Standart 2007, Baek et al. 2008, Selbach et al. 2008) (Figure 1).

Plausible relations for the miRNA-mediated mRNA target degradation rate include linear (mass-law) (Khanin & Vinciotti 2008),

$$\delta(\texttt{miRNA}) = \delta_0\Big(1 + d \cdot \texttt{miRNA}\Big) \tag{3}$$

and Michaelis-Menten type dependency

$$\delta(\texttt{miRNA}) = \delta_0\Big(1 + d \cdot \frac{\texttt{miRNA}}{\gamma + \texttt{miRNA}}\Big). \tag{4}$$

Term $d(\texttt{miRNA})$ (that is equal $d \cdot \texttt{miRNA}$ in (3) and $d \cdot \texttt{miRNA}/(\gamma + \texttt{miRNA})$) in (4)) stands for the miRNA-mediated fold-change in the target mRNA degradation rate relative to the basal degradation rate, δ_0. To account for multiple seeds for the same miRNA (Rajewsky 2006), the above models are easily extended to include the cooperativity, or Hill, coefficient

$$\delta(\texttt{miRNA}) = \delta_0(1 + d \cdot \texttt{miRNA}^h) \tag{5}$$

$$\delta(\texttt{miRNA}) = \delta_0(1 + d \cdot \texttt{miRNA}^h/(\gamma + \texttt{miRNA}^h)), \tag{6}$$

for (3) and (4), respectively. Here $h \geq 1$ is the number of multiple seeds for the same miRNA with optimal spacings of ≤ 40 nt (Grimson et al. 2007, Selbach et al. 2008).

The miRNA-mediated translational repression in (1) can be described by

$$\lambda(\texttt{miRNA}) = \frac{\lambda_0}{\gamma + a \cdot \texttt{miRNA}^h}, \quad h \geq 1, \tag{7}$$

where $a = 1$ when mRNA is a translational target of this specific miRNA and $a = 0$ otherwise.

One miRNA may have many target genes (Krek et al. 2005, Lim et al. 2005, Baek et al. 2008, Selbach et al. 2008). The expression level of the ith target mRNA transcript i is then described by the ODE (Khanin & Higham 2009):

$$\frac{dm_i(t)}{dt} = q_i - \delta_{0i}(1 + d_i(\texttt{miRNA}))m_i(t), \tag{8}$$

$$\frac{dp_i(t)}{dt} = \lambda_i(\texttt{miRNA})m_i(t) - \delta_{Pi}p_i(t). \tag{9}$$

Kinetic parameters q_i, δ_{Pi}, δ_{0i} are gene-specific. In addition, parameters d_i, γ_i, and h_i of the miRNA-mediated downregulation that appear in the functional relations $d_i(\texttt{miRNA})$ and $\lambda_i(\texttt{miRNA})$ are also target specific and depend on the sequence and structure of each mRNA:miRNA base pairing and other recognition elements that have not yet been identified.

The logic of transcriptional regulation by TFs and post-transcriptional regulation by miRNAs appears to be the same (Hobert 2008). Two, or more, miRNAs can regulate a target in different ways. Suppose that two miRNAs, $\texttt{miRNA}_1$ and $\texttt{miRNA}_2$, regulate a target i. Then a SUM-type of regulation may be represented by

$$\delta_i = \delta_{0,i}\Big(1 + d_{i,1}(\texttt{miRNA}_1; h_{i,1}) + d_{i,2}(\texttt{miRNA}_2; h_{i,2})\Big) \tag{10}$$

$$\lambda_i = \frac{\lambda_{0i}}{1 + \texttt{miRNA}_1^{h_{i,1}}/\gamma_{i,1} + \texttt{miRNA}_2^{h_{i,2}}/\gamma_{i,2}}.$$

This is a non-cooperative regulation, wherein the effect of two miRNAs is simply additive. A cooperative SUM-regulation takes place if the presence of two miRNAs results in larger regulation than the sum of each miRNA-mediated regulation. An example of cooperative SUM logic is illustrated in (Grimson et al. 2007) wherein the downregulation of mRNAs in overexpression experiments is larger if the distance between the seeds for transfected miRNA and endogenous miRNAs is within an optimal range. Alternatively, an AND gate logic is relevant if both miRNAs must be present:

$$\delta_i = \delta_{0,i}\Big(1 + d_{i,1}(\texttt{miRNA}_1; h_{i,1}) \cdot d_{i,2}(\texttt{miRNA}_2; h_{i,2})\Big) \tag{11}$$

$$\lambda_i = \frac{\lambda_{0,i}}{1 + \texttt{miRNA}_1^{h_{i,1}} \cdot \texttt{miRNA}_2^{h_{i,2}}/\gamma_i}.$$

Other types of regulation, such as non-exclusive or exclusive OR gates, are also possible and cane be described by similar type of equations.

2.1 Fold-changes of mRNA and proteins at different miRNA levels

The silencing effect of miRNA is estimated experimentally by comparing levels of mRNAs and proteins at two different miRNA levels, usually when miRNA is present ($\mathtt{miRNA} > 0$) or it is absent($\mathtt{miRNA} = 0$). Microarray and proteomic measurements are taken in miRNA mis-expression experiments or across different tissues, cell-lines or conditions. The steady state levels of target mRNAs and proteins ($i = 1 \dots N$) at a given level of the $\mathtt{miRNA}$ can readily be written as

$$m_i = \frac{q_i}{\delta_{0,i}(1 + d_i(\mathtt{miRNA}))} \quad \text{and} \quad p_i = \frac{\lambda_i(\mathtt{miRNA})m_i}{\delta_i^p}. \tag{12}$$

Microarrays, SILAC (Baek et al. 2008) and pSILAC (Selbach et al. 2008) measure fold-changes in mRNAs, protein levels or the number of newly produced proteins under different conditions. The fold-changes for mRNA and protein i are given by

$$FC_i^{\mathrm{mRNA}} := \frac{m_i}{m_i(\mathtt{miRNA} = 0)} = \frac{1}{1 + d_i(\mathtt{miRNA})} \leq 1 \tag{13}$$

$$\begin{aligned} FC_i^{\mathrm{prot}} := \frac{p_i}{p_i(\mathtt{miRNA} = 0)} &= \frac{\lambda_i(\mathtt{miRNA})}{\lambda_i} \frac{m_i}{m_i(\mathtt{miRNA} = 0)} \\ &\leq \frac{m_i}{m_i(\mathtt{miRNA} = 0)} < 1. \end{aligned} \tag{14}$$

The inequality (14) predicts that downregulation of proteins is larger than that of mRNAs, as has indeed been observed for the majority of targets (Selbach et al. 2008). It follows immediately from (13) and (14) that

$$\log_2 FC_i^{\mathrm{prot}} = \log_2 FC_i^{\mathrm{mRNA}} + \Lambda_i, \tag{15}$$

where

$$\Lambda_i = \log_2[\lambda_i(\mathtt{miRNA})] - \log_2[\lambda_i] < 0.$$

Here Λ_i represents direct miRNA-mediated repression of translation that Selbach et al. (2008) computed from experimental data by subtracting the log2 mRNA from the log2 pSILAC fold-changes. Using formula (7) for miRNA-regulated rate of translation, Λ_i can be rewritten as

$$\Lambda_i = -\log_2[1 + \frac{\mathtt{miRNA}^{h_i}}{\gamma_i}],$$

where h_i is the number of seeds acting cooperatively. If miRNA action on the target i results in relatively large translational repression, so that $\mathtt{miRNA}_i^h/\gamma_i \gg 1$, then

$$\Lambda_i \approx -\log_2[\frac{\mathtt{miRNA}^{h_i}}{\gamma_i}] = -h_i \log_2[\mathtt{miRNA}] + \log_2[\gamma_i]. \tag{16}$$

It follows from this formula that, for large negative fold-changes, translational repression measured as log fold-change, is linearly correlated with the number of seeds, as has indeed been observed by comparing pSILAC and microarray data (Selbach et al. 2008). The average number of seeds, plotted as a function of the differences between protein and mRNA fold-changes, exhibits linear decay towards the regime of equal fold-changes (Figure 5c,d; (Selbach et al. 2008)), indicating that in addition to mediating mRNA downregulation, the seed also mediates direct repression of translation rates for hundreds of genes.

Selbach et al. (2008) reported that the slope of the average number of seeds is steeper for pSILAC fold changes than for mRNA fold-changes, suggesting that the multiplicity of a miRNA-binding site in the same 3'UTR exerts a stronger direct effect on protein production than on mRNA levels. This observation is consistent with (15) and (16), since Λ_i is directly proportional to h_i.

We have shown that the above formulas reproduce experimental observations of Selbach et al. (2008). They also suggest that the effect of both mRNA and translational repression has a weak (logarithmic) dependence on the level of miRNA itself. To verify this prediction would require further experimental data from miRNA mis-expression experiments with different levels of miRNA. Our modelling also predicts that fold-changes in mRNA and protein levels do not depend on the initial target level. So targets that are expressed at different levels are downregulated to the same extent provided the kinetic parameters of the miRNA regulation are the same. Indeed, the average protein fold-changes (pSILAC data from Selbach *et al*, 2008) for targets with seeds in their 3'UTRs as functions of their mRNA levels at control do not exhibit any pattern in protein fold-changes for a large range of control intensities (Khanin & Higham 2009, Figure??).

3 Non-steady-state behaviour

It is also informative to consider non-steady state behaviour of the model (1)–(2). Fitting the model to temporal microarray and pSILAC (or SILAC) data upon miRNA transfection/knock-down will yield kinetics of the miRNA-mediated effect on a target gene's degradation and translation rates. Indeed, Khanin & Vinciotti (2008) demonstrated that it is possible to infer kinetic parameters of miRNA-mediated mRNA degradation using temporal gene expression data from overexpression experiments. Here we explore scenarios of miRNA mis-expression experiments wherein miRNA levels quickly change to a new constant level due to transfection or knock-down.

3.1 MicroRNA levels are constant

If the miRNA level in a cell-line or tissue does not change much with time post-transfection (or post-knock-down), the value of $\mathtt{miRNA}$ in the system (1)–(2) can be fixed at some arbitrary level and a closed-form solution for each target mRNA for the transfection experiment can readily be written as:

$$\begin{aligned} m_i(t) &= m_i^0 e^{-\delta_i(\mathtt{miRNA})t} + m_i(\mathtt{miRNA})(1 - e^{-\delta_i(\mathtt{miRNA})}), && (17)\\ p_i(t) &= p_i^0 e^{-\delta_{pi}t} + p_i(\mathtt{miRNA})(1 - e^{-\delta_{pi}t}) && (18)\\ &+ \frac{\lambda(\mathtt{miRNA})q_i}{\delta_{0i}\delta_i(\mathtt{miRNA})}\frac{\delta_i(\mathtt{miRNA}) - \delta_{0i}}{\delta_{pi} - \delta_i(\mathtt{miRNA})}(e^{-\delta_i(\mathtt{miRNA})t} - e^{-\delta_{pi}t}), \end{aligned}$$

where the initial levels of target mRNA and protein i are given by

$$m_i^0 = m_i(\mathtt{miRNA} = 0) = \frac{q_i}{\delta_{0i}}, \quad p_i^0 = p_i(\mathtt{miRNA} = 0) = \frac{\lambda_i}{\delta_{pi}}m_i^0 \qquad (19)$$

and their levels at new miRNA level are

$$m_i(\mathtt{miRNA}) = \frac{q_i}{\delta_i(\mathtt{miRNA})}, \; p_i(\mathtt{miRNA}) = \frac{\lambda_i(\mathtt{miRNA})}{\delta_{pi}}m_i(\mathtt{miRNA}) \qquad (20)$$

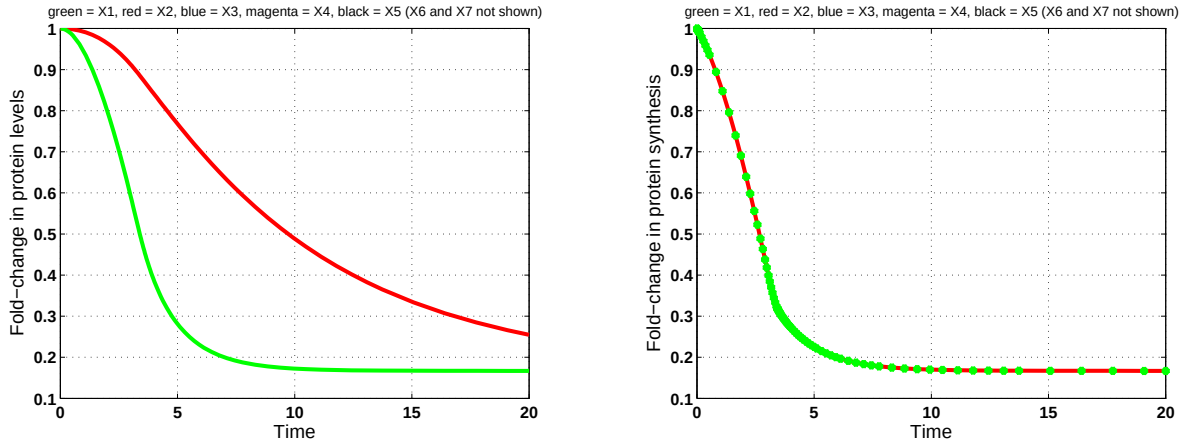
The above equations can be used for estimating time-responses of different targets to miRNA transfection. It is clear that the time-scale for introducing miRNA-mediated repression is determined by either δ_{pi} or $\delta_i(\mathtt{miRNA})$, whichever is slower. Therefore, the levels of high-turnover proteins (large δ_{pi}) will change rapidly whereas stable proteins (low δ_{pi}) will be affected later, as seen in Figure 2. If the measurements of protein levels by standard techniques

such as SILAC are done at times less than 10–15h post-transfection, then erroneous conclusions will be reached that the "black" protein is downregulated to a lesser degree than the "grey" protein. Thus, to assess endogenous regulation of mRNA translation by miRNAs, a technique such as pulsed SILAC is needed to measure directly genome-wide changes in protein synthesis shortly after changes in miRNA expression (Selbach et al. 2008).

Similarly, the time-course of de-repression (removing miRNA-mediated repression) can be found by solving equations (1)–(2) with knocking-down miRNA at $t = 0$ ($\texttt{miRNA} = 0$) and initial conditions $\text{mRNA}(0) = m_i(\texttt{miRNA})$ and $\text{prot}(0) = p_i(\texttt{miRNA})$. The time-responses for different types of mRNAs and proteins can be estimated and compared with removing TF-mediated repression, similar to the case of small RNAs as has been done by Shimoni et al. (2007). The simple model (1)–(2) adequately describes experimental observations from miRNA transfections and knock-downs as shown above. This model, however, does not include miRNA-mediated relocation of mRNAs into P-bodies that can quickly be released into the cytoplasm (Bhattacharyya et al. 2006). This suggests that to account for quick miRNA-mediated repression and de-repression (Hobert 2008), as occurs when the miRNA profile drastically changes in a short time-window in development processes or in response to stress, the step of relocation/release of miRNA:mRNA complexes from P-bodies and their subsequent translation is required.

3.2 MicroRNA levels are changing

It is quite plausible that miRNA levels are influenced by TFs (Shalgi et al. 2007), or by other miRNAs (Tuccoli et al. 2006). In addition, miRNAs can decay before they are incorporated into the stable RISC complexes or degrade in the process of a few cycles of mRNA binding.The latter can be accounted for, for example, by introducing a global parameter that represents the probability for a miRNA to be co-degraded with the mRNA in the processed state. Additionally, miRNA molecules, incorporated into the RISC complexes, are sequestered into the target mRNA:miRNA complexes, subsequently translocated to the P-bodies. This can be modelled as a multi-step process, as discussed in section 5. Some of the mRNA:miRNA complexes might be stored in the P-bodies to be released in response to stress (Bhattacharyya et al. 2006). In miRNA overexpression experiments, the miRNA



(a) (b)

Figure 2: Protein profiles post-transfection with miRNA: (a)-(b) miRNA experiment: "black" protein has degradation rate that is ten-fold larger then the that of "grey" protein.

levels can decrease due to cell growth/division, and natural miRNA decay. The effects that cause miRNA levels to decrease can be lumped together and considered as a linear decay process of miRNA with the rate δ_m (Khanin & Vinciotti 2008):

$$\frac{d\mathtt{miRNA}(t)}{dt} = s(t) - \delta_m \mathtt{miRNA}(t). \tag{21}$$

Here $s(t)$ is the rate of miRNA transcription that might depend on various TFs. In cell-lines and tissues where a specific miRNA is present, its level can be approximated by s/δ_m. In miRNA transfection experiments, the miRNA level is at its maximum level at the initial (transfection) time: $\mathtt{miRNA}(0) = 1$. Assuming no production of the miRNA in the cell-line or tissue where it is normally not present, $s(t) = 0$, the miRNA temporal profile is determined by just one parameter, δ_m: $\mathtt{miRNA}(t) = e^{-\delta_m t}$. Interestingly, the majority of downregulated target mRNA expression profiles measured for up to 120h in miR124a transfection show upregulation in the last time-point(s) (Wang & Wang 2006), thus suggesting that the subsequent upregulation of the target mRNAs is due to a decrease in the level of the regulator miRNA (Khanin & Vinciotti 2008).

3.3 Reconstructing kinetics of mRNA-regulated Single Input Motif from high-throughput data

Kinetic parameters of the miRNA-mediated target downregulation are difficult to measure experimentally, but they can be inferred via time-course high-throughput datasets from miRNA mis-expression experiments using a minimal model (1)–(2). The first attempt in this direction has been done by Khanin & Vinciotti (2008), where the authors apply the model to temporal gene expression data from a miRNA transfection experiment (Wang & Wang 2006). Similarly, kinetics of miRNA-mediated translational repression can be estimated from protein measurements.

Let us consider the simplest miRNA-regulated gene circuit that involves one miRNA post-transcriptionally acting on its numerous targets. This structure is similar to the so-called Single Input Motif (SIM) that is common in transcription networks (Shen-Orr et al. 2002). A miRNA-SIM has larger number of targets on average than a TF-SIM. Both types of SIMs are condition-specific because many targets are presumably subject to control by other miRNAs or TFs whose levels do not change in the course of the experiment. Their effects are implicitly incorporated in the basal rate-constants of transcription and degradation of target mRNAs and proteins.

Kinetic parameters of the SIM targets $\{q_i, \delta_{i0}, \lambda_i, \delta_{pi}, \delta_i(\texttt{miRNA}), \lambda(\texttt{miRNA})\}_{i=1}^{N}$, including the unknown profile of the miRNA master regulator that is common to all targets, can be reconstructed from the high-throughput data (Khanin et al. 2006). One way of estimating parameters of miRNA-mediated mRNA degradation from microarray data involving miRNA overexpression is by the maximum likelihood procedure (Khanin & Vinciotti 2008). This method applied to the time-course post-transfection with miRNA124a yielded a miRNA124a half-life of $29h$ (95% confidence bounds $(26h, 50h)$). This estimate incorporates the effects of free miRNA decay, its sequestering into P-bodies as well as cell growth (and thereby miRNA dilution). In addition, it has been observed in this study that the miRNA downregulating effect on the target mRNA degradation rates can adequately be described by either a non-linear (4) or a linear model (3). In order to distinguish between the two models, miRNA mis-expression (overexpression and silencing) experiments are required where the miRNA levels can be measured. A model that takes into account multiple sites for the same miRNA on the 3'UTR of the target mRNAs (5), gives a better fit to some mRNA profiles, so the number of active seeds, h, can also be estimated from the data. Experiments wherein

the mRNA expression is measured at different levels of miRNA would be particularly helpful in determining the miRNA dosage-dependent effect on the target downregulation. Khanin & Vinciotti (2008) estimated effective miRNA-mediated fold-change increase in each target mRNA degradation rate, and the reconstructed basal decay rates of target mRNAs in this study have a very good correspondence with experimental measurements from an independent study (Yang et al. 2003), thereby giving a strong support for this modelling approach. These methods, with extended modelling assumptions, and other optimization and inference techniques, such as Bayesian inference (Rogers et al. 2007), can be applied to new experimental datasets and will yield kinetic information on miRNA-regulation, as well as miRNA time-course and biogenesis.

4 Models of miRNA-mediated network motifs

As different types of high-throughput data accumulate, it is natural to integrate them together with experimentally verified regulatory relationships as well as those obtained from bioinformatics tools and existing functional genomics data (Rajewsky 2006). An initial effort in this direction has been published for nematodes (Lall et al. 2006). There is now a growing number of papers that explore the "wiring" of miRNA regulatory relationships together with known transcriptional regulatory interactions (Cui et al. 2007, Shalgi et al. 2007, Yu et al. 2008), signal transduction networks (Cui et al. 2006) and protein-protein interactions networks (Liang & Li 2007, Hsu et al. 2008). These networks are constructed by using computational predictions for miRNA targets and transcription factor binding sites. It appears that a very large number (up to 43%) of human genes are under combined transcriptional and post-transcriptional regulation (Shalgi et al. 2007). The true number of human genes that are subject to a dual TF and miRNA regulation is probably even higher considering the fact that the collection of mammalian miRNAs is yet incomplete.

Studies of network structures that involve miRNAs and TFs started by considering just a few experimentally confirmed cases (Hornstein & Shomron 2006). Some network motifs have been experimentally found in *C.elegans*, notably a double-negative feedback loop (Hobert 2004). More recently, com-

posite feedback loops in which a TF that controls a miRNA is itself regulated by that same miRNA have been shown to be over-represented (Martinez et al. 2008). TFs that control cell proliferation and apoptosis (Myc and E2F), and crucial pathways, such as the p53 master network, have been found to be under tight control of several miRNAs (Aguda et al. 2008, Sinha et al. 2008). The goal now is to identify the over-represented motifs in large networks and to understand how functionality is related to structure (Shalgi et al. 2007, Tsang et al. 2007, Yu et al. 2008).

Initial kinetic models of gene regulatory circuits with feedback between miRNAs and TFs have recently appeared (Aguda et al. 2008, Yu et al. 2008, Zhdanov 2009). In these studies, a TF is one of the targets of the miRNA under consideration, whose transcription rate, $s(t)$ (eqn. 21) is regulated, positively or negatively, by the same TF (here denoted by P with cooperativity n):

$$s(t) = \beta \frac{P^n}{(\gamma + P)^n} \quad \text{for } \textit{activator}; \quad s(t) = \frac{\beta}{(\gamma + P)^n} \quad \text{for } \textit{repressor}. \tag{22}$$

Mathematical models of the basic miRNA-TFs structures demonstrate their complex and intricate behaviour. The TF-miRNA feedback circuit can operate as the simplest biological switch (Zhdanov 2009), potentially changing levels of a large number of other targets. Feedback loops often include autoregulation of TF (e.g. a cancer network that comprises miR-17-92 cluster, E2F and Myc (Aguda et al. 2008)). Such autoregulated feedback loops exhibit changes in the steady-state levels of TF and miRNA that go in the same direction (Aguda et al. 2008). This agrees with experimental observations on Myc in miR-17-92 cluster in various tumors, but is somewhat counter-intuitive, as one might expect that a mRNA/protein that is a target of a miRNA and the miRNA are expressed reciprocally in different tissues—this has indeed been shown to be the case for many targets (Sood et al. 2006).

The case of two TFs regulating each other with one miRNA regulating both of them has been shown to be the most significant overrepresented network motif in a human regulatory network (Yu et al. 2008). Mathematical modelling demonstrated that miRNA stabilizes mutual regulation of two TFs to resist perturbations. On the other hand, such a motif has the ability to convert a transient stimulus into a stable and irreversible response. Mathematical modelling of the fundamental structures has already demonstrated that the basic repressive function of miRNAs when combined with other regulatory factors can build up more complex and higher-order functions such as

fine-tuning, canalization (Hornstein & Shomron 2006) and multi-dimensional switches.

It must be noted, however, that current models of miRNA-TF feedback circuits do not take into account the potential impact of numerous other miRNA targets on the circuit behaviour (Aguda et al. 2008, Yu et al. 2008). More realistic models of such circuits should be extended to include the potential effect of multiple miRNA targets (Zhdanov 2009, Khanin & Higham 2009). Full models that study functionalities of miRNA-TF network motifs should also take into account all possible modes of miRNA-mediated target regulation, i.e. mRNA destabilization and/or repression of translation, and multiple seeds for a miRNA (cooperativity in equation (5)). According to a recent study (Yu et al. 2008), there exist two classes of miRNAs: miRNAs in one class are regulated by TFs, while the other class of miRNAs regulate TFs. Modelling the behaviour of both miRNA classes requires detailed consideration of how inputs from different types of regulators, miRNAs or TFs, exert their regulatory effect (equations (10) and (11)). Another feature of the TF and miRNA regulatory circuits that may be significant is the time delay between production and regulatory action (Aguda et al. 2008, Xie et al. 2007).

Modelling of networked structures is crucial in unravelling miRNA functionality and its relationship with other regulatory factors. Related questions include whether miRNA-driven switches or double-negative feedback loops (Hobert 2004) have any advantage over similar structures that are controlled on transcriptional level. TF-driven switches and double-negative feedback loops have been theoretically and computationally studied in great detail by several authors. Similar studies of basic miRNA-mediated switches will undoubtedly be performed in the near future, and will reveal the commonalities and differences between these structures. It seems likely that interwoven regulatory combinations of miRNAs and TFs yield robust multidimensional switches that frequently occur in differentiation (RK, unpublished observations).

Below we will briefly discuss how the simple model (1)–(2) can be extended to include the binding of miRNA to its target mRNAs (Levine et al. 2007b, Khanin & Higham 2009). This brings about miRNA-mediated target *cross-talk* if miRNA degrades after a few cycles target mRNA binding.

5 A multi-step model: including miRNA binding to target mRNA

The two-step model of (Levine et al. 2007b) is based on plausible biological assumptions: the binding of miRNA to the mRNA promotes a secondary process (e.g. ribosome run-off or deadenylation) that ultimately leads to mRNA accumulation in its processed state, perhaps in P-bodies. The authors show that the target mRNA and protein levels may be tuned by target-specific parameters while global effectors may alter this behavior for some, but not all, miRNA targets in the cell. However, this model erroneously predicts that the fold-changes in protein levels can not be higher than those of corresponding mRNAs (eqn. 2 in Levine et al. (2007b)), contrary to experimental findings (Selbach et al. 2008). This is due to their assumption that proteins are produced at equal rates from both free and microRNA-bound mRNAs.

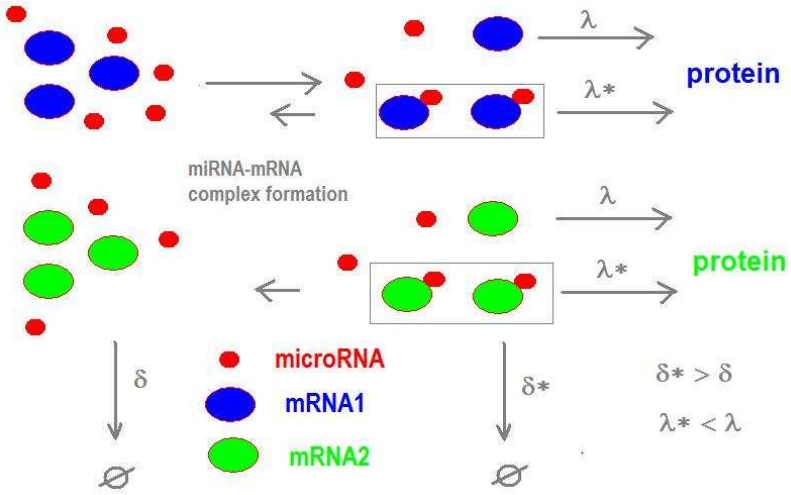


Figure 3: Cartoon of microRNA bindings to mRNA targets

Khanin & Higham (2009) developed a model that takes into account the miRNA binding to mRNAs, and gives results that are consistent with miRNA mis-expression experiments (see also (Khanin & Higham 2007)). Here we will

outline the model assumptions, display the ODE system, and briefly present the results.

To set up a model, consider a system where a single type of miRNA targets several different types of mRNA molecules, m_i. We assume that each type of mRNA is produced with its own transcription rate q_i and decays with its own rate δ_i. The miRNA itself is being produced in the cell with a rate p_m and decays with a rate δ_m. The model allows mRNA and miRNA to form a complex, $\texttt{miRNA} \cdot m_i$, with a forward rate β_i and a reverse rate β_i^-. The complex $\texttt{miRNA} \cdot m_i$ decays at a rate $\delta_i^\star$. Proteins, p_i, are being translated at a rate λ_i from free mRNAs, m_i, and with a rate $\lambda_i^\star$ from the complexes, $\texttt{miRNA} \cdot m_i$, and degrade at a rate δ_i^p. The key downregulating property of the miRNA is introduced by two constraints:

- $\delta_i^\star \geq \delta_i$, so that the complex degrades faster than free mRNA, and/or
- $\lambda_i^\star \geq \lambda_i$, so that the complex produces protein more slowly than free mRNA.

The ratios $\delta_i^\star/\delta_i$ and $\lambda_i^\star/\lambda_i$ will depend on specific target mRNA and miRNA base-pairing in and around the seed region. We further suppose that when the complex degrades, a fixed proportion, $0 \leq \kappa \leq 1$, of the miRNA returns to the pool.

In the case where there are two targets, the ODE model may be written

$$\begin{aligned}
\frac{dm_1}{dt} &= q_1 - \delta_1 m_1 - \beta_1 m_1 \texttt{miRNA} + \beta_1^- \texttt{miRNA} \cdot m_1 \\
\frac{dp_1}{dt} &= \lambda_1 m_1 - \delta_1^p p_1 + \lambda_1^\star \texttt{miRNA} \cdot m_1 \\
\frac{dm_2}{dt} &= q_2 - \delta_2 m_2 - \beta_2 m_2 \texttt{miRNA} + \beta_2^- \texttt{miRNA} \cdot m_2 \\
\frac{dp_2}{dt} &= \lambda_2 m_2 - \delta_2^p p_2 + \lambda_2^\star \texttt{miRNA} \cdot m_2 \\
\frac{d\texttt{miRNA}}{dt} &= p_m - \delta_m \texttt{miRNA} - \beta_1 m_1 \texttt{miRNA} + \beta_1^- \texttt{miRNA} \cdot m_1 \\
&\quad - \beta_2 m_2 \texttt{miRNA} + \beta_2^- \texttt{miRNA} \cdot m_2 + \delta_1^\star q \texttt{miRNA} \cdot m_1 + \delta_2^\star q \texttt{miRNA} \cdot m_2 \\
\frac{d\texttt{miRNA} \cdot m_1}{dt} &= \beta_1 m_1 \texttt{miRNA} - \beta_1^- \texttt{miRNA} \cdot m_1 - \delta_1^\star \texttt{miRNA} \cdot m_1 \\
\frac{d\texttt{miRNA} \cdot m_1}{dt} &= \beta_1 m_1 \texttt{miRNA} - \beta_1^- \texttt{miRNA} \cdot m_1 - \delta_1^\star \texttt{miRNA} \cdot m_1.
\end{aligned}$$

This generalizes readily to any number of targets; see (Khanin & Higham 2009).

Although this nonlinear ODE system cannot be solved analytically, it is possible to analyse the steady state behaviour. It can be shown that the $\kappa = 1$ regime completely uncouples the targets, while $0 \leq \kappa < 1$ introduces coupling into the system, wherein the level of achieved miRNA-mediated repression is dependent on the amount of miRNA itself, the expression level of the target under consideration and levels of other targets Doench & Sharp (2004). Indeed, miRNA degradation after several rounds of binding to target mRNA results in miRNA-mediated target cross-talk, when changing the level of one target has an effect on the level of other target(s). Increase in the level of one target can be caused by external signal, or by some feedback in the circuit itself, wherein one of the targets is a transcription factor for another one. Additionally, as discussed above, the feedback between target TFs in the circuit and miRNA can result in changing the levels of the miRNA itself.

To illustrate these effects, in Figure 4 we give computational solutions to the full ODE model. We show output over a time interval $0 \leq t \leq 10$, where at time zero, each species was set to a level of 10. The figure shows the level of target one protein $p_1(t)$, and the level of miRNA, $\texttt{miRNA}(t)$. We set $q_2 = 1$ in the upper picture and and $q_2 = 25$ in the lower picture. We see from the figure that increasing q_2, that is, raising the transcription rate fo the second target, causes the level of target one protein to rise. This is explained by the decreased availability of miRNA. The repressive effect of miRNA on target one has been reduced because more miRNA is binding to the over-abundant target two.

The figure illustrates that levels of mRNAs that are targets of the same miRNA are interdependent, via the level of miRNA that affects them. If the levels of one or several mRNAs increases due to transcriptional control, the miRNA may become limiting and its downregulating effect on the rest of the targets will be substantially decreased. If it is crucial to keep other targets at a low level, then additional controls at either transcriptional or post-transcriptional level (by combinatorial regulation with another miRNA) are needed. Indeed, a recent study strongly suggested that coordinated transcriptional and post-transcriptional (p53 and miRNAs mediated) networks are an integral part of tumorigenesis (Sinha et al. 2008).

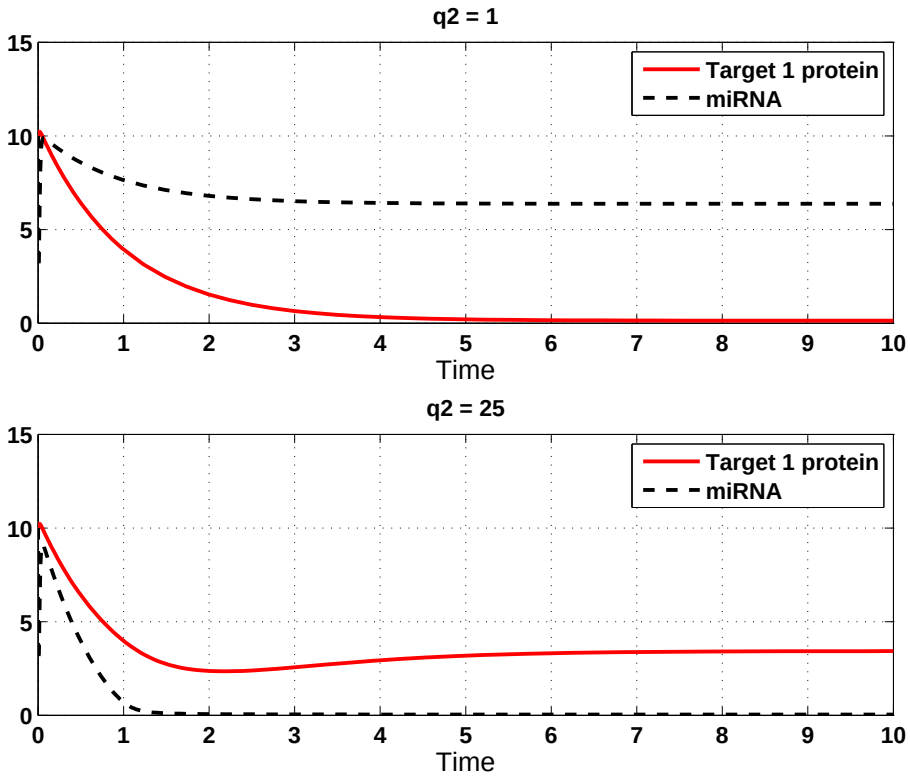


Figure 4: Levels of target one protein and miRNA. In the lower picture, where $q_2 = 25$, target two mRNA production is much greater and target one protein production has indirectly benefited. for Parameter values $q_1 = 5$, $\delta_1 = 1$, $\delta_2 = 1$, $b_1 = 50$, $b_2 = 50$, $b_1^- = 0.1$, $b_2^- = 0.1$, $\lambda_1 = 2$, $\lambda_2 = 1$, $\delta_1^+ = 1$, $\delta_2^+ = 1$, $\lambda_1^\star = 2$, $\lambda_2^\star = 2$, $p_m = 10$, $\delta_m = 1.1$, $\delta_1^\star = 100$, $\delta_2^\star = 100$ and $\kappa = 0.5$.

Extrapolating from our simple illustration of two targets to the case where several types of miRNA interact with multiple targets, it becomes clear that the problem of inferring regulatory relationships directly from mRNA expression data alone will generally be infeasible due to the high level of cross-talk.

The multi-step model for a large number of targets includes many different parameters. It is yet to be determined whether rate-constants for miRNA-mRNA complex formation/dissociation are target specific or whether it is plausible to assume that the kinetics of the translocation to/from P-bodies is governed by global parameters that are determined by cell condition (Levine et al. 2007b).

The above multi-step model was not designed to address the molecular mechanism of miRNA action. A very promising step in this direction has recently been undertaken by Nissan & Parker (2008), who examined by means of computational analysis the effect of miRNA on different steps in translation. Kinetic analysis of their model with the rate-constants carefully estimated from experimental data demonstrate that a miRNA will have a modest effect on the overall rate of protein production from a specific target mRNA if the step it affects is not rate-determining. Their model is consistent with the suggestion that miRNAs may primarily repress translational initiation at a late step. However, the authors demonstrate that the experimental observations used to argue for this suggestion are open for alternative interpretations (Nissan & Parker 2008).

6 Future prospects

In this review, we described recent progress in computational and mathematical modelling of post-transcriptional gene regulation by miRNAs. We can divide current approaches into three types or classes. The first approach deals with computational prediction of miRNA targets. A growing number of algorithms predict miRNA sites on the targets taking into account miRNA-mRNA base-pairing and its secondary structures, seed conservation among species and other sequence characteristics, including AU-richness and proximity of various RNA motifs. Despite various underlying and additional modelling assumptions, all target prediction tools are clearly `sequence`-based.

Another approach that is gaining momentum is integration of available predictions from the `sequence`-based methods together with other regulatory relationships, such as transcription factors and their targets, into a `network`-based approach. Unlike purely transcriptional regulatory networks studied by many groups, miRNA–TF networks are two dimensional, as each target (node) can be regulated on two different levels: transcriptional and post-transcriptional. In addition, regulators in this network, TFs and miRNAs, mutually regulate each other. Moverover, post-transcriptional regulation involves miRNA-mediated destabilization of target mRNA and/or translational repression, and the links connecting the two would ideally indicate the mode of regulation. Study of two-dimensional, directed networks clearly requires new tools for description, representation, visualization and efficient search algorithms for over-represented motifs.

In this chapter, we focused on a third class of models, based on `expression` levels. Although we restricted ourselves to kinetic modelling of miRNA-mediated gene regulation using ordinary differential equations; we note that equations can easily be translated into stochastic form in order to study extrinsic and intrinsic noise and the cases of low number of molecules (Higham 2007, M.Marba 2009).

With very few exceptions, `sequence`, `network` and `expression` approaches used in computational studies of miRNA-mediated regulation currently go pretty much in parallel without much much feedback between them. Clearly, both `network` and `expression` approaches require predictions from `sequence` algorithms. In addition, ODE models of over-represented network motifs are being built and studied. But can the results from the`network` and ODE based approaches improve the target prediction tools? In our view, they can. Future experimental data should be interpreted by taking into account not only sequences of the participating mRNAs and proteins, as has been successfully done in the past (Baek et al. 2008, Selbach et al. 2008). Target prediction and verification can benefit from including information on expression levels of miRNAs and mRNAs as well as details of regulatory miRNA–TF structures. On one hand, Khanin & Higham (2009) have shown theoretically and verified on pSILAC datasets that targets that are expressed at different levels are downregulated to the same extent (i.e. their fold-changes are equal) provided the kinetic parameters of the miRNA regulation are the same. On the other hand, the number of false-positives in lowly expressed mRNAs at control is likely to be considerably larger than for those mRNAs that are expressed at higher levels (RK, unpublished observations on pSILAC data).

Dynamic modelling of network structures and pathways from a consortium of datasets that will become available in the next 5–10 years might therefore point at some additional features that ought to be included in target prediction algorithms. An important contribution would be to identify specific conditions when certain miRNA sites are active, as they require cooperative action from other nearby miRNA sites or RNA-binding proteins, thereby reducing the fraction of false-positives from the `sequence` based predictions. The avalanche of new data sets can be harnessed by integrating different types of data and studying them computationally, by taking into account sequences, regulatory relationships, and kinetics simultaneously, in our effort to elucidate mechanisms and functions of miRNAs.

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